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13. Abstract *(Maximum 200 Words)* Androgens play important roles in the differentiation, development and maintenance of male reproductive functions, as well as in the etiology of prostate cancer. The biological effects of androgens are believed to be mediated through the intracellular androgen receptor (AR), which belongs to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors. Like other NRs, the actions of AR are subject to modulation, either positively or negatively, by an increasing number of co-regulatory proteins, termed coactivators or corepressors. Coactivators are believed to function either as bridging factors between receptors and basal transcription machinery to enhance recruitment of the basal transcription machinery and/or as factors that have capacity to actively remodel repressive chromatin. The purpose of this research is to study the mechanism by which coactivators modulate AR activity in chromatin, the physiological template of transcriptional regulation. In this progress report, we report that we have analyzed how SRC family coactivators and p300 modulate AR activity in the context of chromatin using Xenopus oocyte as a model system. We demonstrate that p300 requires both its histone acetyltransferase activity and interaction with SRC family coactivators to stimulate AR activity. Surprisingly, we also observed that AR possesses an intrinsic hormone-independent activity when AR is overexpressed. Coactivators such as SRC and p300 can further augment this hormone-independent activity by AR. We propose that this intrinsic hormone-independent activity could be relevant to the function of AR in hormone-independent prostate cancer.

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Introduction

Androgens play important roles in the differentiation, development and maintenance of male reproductive functions, as well as in the etiology of prostate cancer. The biological effects of androgens are believed to be mediated through the intracellular androgen receptor (AR), which belongs to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors (1, 2). Like other NRs, AR is composed of distinct functional domains that include an amino-terminal domain that contains one or more trans-activation functions (AF1), a highly conserved DNA binding domain (DBD) and a multi-functional carboxyl-terminal ligand binding domain (LBD) that is involved in homo- or hetero-dimerization of the receptors, binding of specific ligands, and contains a ligand-dependent activation function (AF2) (7, 30).

Early studies indicate that in the absence of ligands, AR resides primarily in cytoplasm and is believed to associate with heat shock proteins in an inactive state (14, 28). Binding of ligand to AR is believed to trigger a series of events, including a change of conformation, translocation from the cytoplasm to the nucleus, and subsequent binding to specific promoter response elements, which eventually leads to activation or repression of its target genes (30). Like other NRs, research in the last several years has revealed an increasingly complex picture of the mechanism of transcriptional regulation by AR. The actions of AR are subject to modulation, either positively or negatively, by an increasing number of co-regulatory proteins, termed coactivators or corepressors (8, 23). Coactivators are believed to function either as bridging factors between receptors and basal transcription machinery to enhance recruitment of the basal transcription machinery and/or as factors that have capacity to actively remodel repressive chromatin (8). While some coactivators such as ARA70 (36) or FHL2 (25) may be specific for AR, many of the coactivators identified so far, including SRC family coactivators, CBP, p300, PCAF and TRAP/DRIP/ARC complexes are generic to NRs [for review, see (8, 24)]. Importantly, many coactivators possess intrinsic histone acetyltransferase activity. In contrast, corepressors such as SMRT and NCoR are found to associate with histone deacetylases in large protein complexes (10, 12, 21, 26). These findings provide a strong molecular connection between the modification of chromatin structure and transcriptional regulation by NRs. Indeed, a conceptual advance in our understanding of transcription control over the last several years is the recognition of chromatin structure as an integral component of transcriptional regulation in eukaryotic cells (29). In comparison to other NRs such as thyroid hormone receptor (TR), glucocorticoid receptor (GR), progesterone receptor (PR) and estrogen receptor (ER), little is known about how AR regulates transcription in the context of chromatin.

Uniquely among steroid hormone receptors, the hormone-dependent AF2 activity of AR is elusive. Deletion of the LBD generates an AR molecule with constitutive activity that in many transcription assays is equivalent to the activity of the full-length AR in the presence of ligands, whereas deletion of the N-terminal AF1 domain usually results in an AR molecule with low or no detectable activity even in the presence of ligands (15, 28). These observations suggest that AF1 contributes most, if not all, the activity of AR. Consistent with this idea, several studies indicate that the AF1 domain mediates primarily the interaction between the SRC family coactivators and liganded AR (1, 4, 11). However, those observations also raise the question as, if the transcriptional activity of AR is primarily derived from its AF1 activity, why AR is transcriptionally inactive in the absence of androgens. One simple explanation comes from the

fact that AR is primarily located in cytoplasm in the absence of hormones. Such subcellular localization thus prevents unliganded AR to be engaged in transcription, although its AF1 domain could be potentially transcriptionally active in the absence of androgens. A second, but not mutually exclusive, explanation comes from the recent observation that a ligand-dependent intra-molecular interaction between AF1 and AF2 domains is essential for activation of the AR AF1 activity (13, 19).

In addition to activation by binding of androgens, AR can be activated in the absence of androgens in different cell lines by growth factors such as IGF-1 (insulin-like growth factor) and EGF (epidermal growth factor) or chemicals that directly activate the protein kinase A signaling pathway (5, 27). The mechanism of such ligand-independent activation is not clear yet, but likely to involve in phosphorylation of AR and/or its associated proteins.

Androgens are known to play a crucial role in the occurrence and progression of prostate cancer. Recent research in prostate cancer provides evidence for the existence of ligand-independent activity of AR. Patients with advanced prostate cancer are usually subjected to hormonal therapy by either androgen deprivation and/or blockade of AR with anti-androgens (16). These treatments are beneficial in the early stages of cancer but eventually lead to relapse of androgen-insensitive cancers. Paradoxically, many hormone-insensitive prostate cancers are found to be positive for both AR as well as the gene products that are regulated by AR (6, 9), suggesting that AR may still remain functionally active and thus contribute to the progression of androgen-independent prostate cancer. While mutations in AR may lead to activation of AR in the absence of ligands or a change in its hormone-specificity, recent studies indicate that mutations in AR are rare even in hormone-insensitive cancers (16, 17). Instead, the amplification and consequent overexpression of the wild-type AR gene appears to be the most common event found in hormone-refractory prostate cancer (16). These observations have led to the hypothesis that over-expression of AR and its subsequent activation by growth factor-mediated cross-talk pathways could lead to the ligand-independent activation of AR in hormone-insensitive prostate cancer. However, it is not known whether overexpression of AR alone is able to activate transcription in the absence of cross-talk pathways.

An important question related to the issue of the hormone-independent activity is whether AR can bind to an androgen response element (ARE) in the absence of ligand. Although ligand is usually required for androgen-dependent transcription activation since AR is located primarily in cytoplasm in the absence of ligand, the fact that AR can be activated by other signaling pathways in the absence of ligand argues that AR has the capacity to bind DNA in a ligand-independent manner. So far, *in vitro* gel shift assays have yielded conflicting results on this subject. In one case *In vitro* translated AR or AR produced in insect cells is capable of binding to AREs *in vitro* in the absence of ligand (18, 22, 35), whereas in other cases pretreatment with ligand is required for DNA binding *in vitro*(32). The controversy over whether AR can bind DNA in the absence of ligands *in vitro* is at least partly due to the technical difficulty in producing sufficient amounts of recombinant unliganded AR proteins and further complicated by the fact that AR appears to have an intrinsic weak DNA binding activity.

Our previous work and that of others have established *Xenopus* oocytes as an excellent model system for studies of transcriptional regulation by NRs in the context of chromatin (33,

34). *Xenopus* oocytes contain a large storage of factors required for transcription and both histones and non-histone proteins required for chromatin assembly. *Xenopus* oocytes are well suited for introduction of DNA, mRNA or proteins through microinjection. Introduction of DNA into the nucleus of *Xenopus* oocytes through microinjection allows the assembly of injected DNA into chromatin through two different pathways depending upon the type of DNA injected. While microinjection of DNA templates either as single-stranded (ss) or double-stranded (ds) DNA into *Xenopus* oocyte nucleus leads to the assembly of both DNA templates into chromatin, the chromatin template resulted from injection of ssDNA is more refractory to basal transcription than that generated by dsDNA. This is most likely due to the fact that the ssDNA injected into *Xenopus* oocyte nucleus is rapidly converted into dsDNA through the synthesis of the complementary strand and the resulting dsDNA is assembled into chromatin within 30 minutes after injection in a process coupled to the synthesis of the complementary strand (replication-coupled assembly pathway) (2, 34), which mimics the chromatin assembly process during S phase in cell cycle.

The purpose of this research is to study the molecular mechanisms by which AR regulates transcription in the context of chromatin. In our original proposal, we proposed to use Xenopus oocytes as a model system and to also establish a chromatin-based cell-free transcription system to study transcriptional regulation by AR and its coactivators in the context of chromatin. In this progress report, I summarize significant progress we have made so far on some of the tasks and also unexpected problems in others.

Body

The long-term objective of our original proposal is to understand the molecular mechanisms by which AR regulates transcription in prostate cancer. Since transcriptional regulation in eukaryotic cells takes place at the level of chromatin, we proposed to establish both an in vivo and in vitro model system to study how AR and its coactivators regulate transcription in the context of chromatin. Toward this goal, two specific aims were proposed:

Aim 1. To establish a Xenopus oocyte-based and a cell-free transcription systems to study the mechanisms by which AR and its coactivators function in the context of chromatin.

Aim 2. To study the transcriptional profile of the AR mutant (877Thr-Ala) frequently found in advanced human prostate cancer.

Significant progress has been achieved on most of the proposed first year tasks for our proposal. However, as scientific research is often not progress as planned, one can see from this first year report that we have achieved tremendous progress in some tasks but not the other. Next I will summarize the progress on each proposed task.

Task 1. Generate the constructs for and express the expression of p300 and ARA70 in Xenopus oocytes

Although human p300 is large protein of 2414 amino acids, we have successfully cloned the cDNA encoding the human p300 into pSP64(polyA) vector. The expression of human p300 in Xenopus oocytes was achieved by synthesis the p300 mRNA in vitro and subsequent injection of the mRNA into Xenopus oocytes. The expression of p300 in Xenopus oocytes after injection of its mRNA was confirmed by western blotting using a p300 specific antibody (Fig. 1B). The p300 expressed in Xenopus oocytes was functionally active judging from its intrinsic histone acetyltransferase activity (Fig. 1B) and its ability to stimulate AR-dependent activation in Xenopus oocytes (see Fig. 7 and 9). Surprisingly, so far we have not been able to express ARA70 in Xenopus oocytes. Similar to the approach for p300, we cloned cDNA encoding ARA70 into the pSP64(polyA) vector and verified the ARA70 sequence in the resulting construct by DNA sequencing. We are currently trying to improve the expression of ARA70 in Xenopus oocytes by optimizing the codon usage at the N-terminal region of the ARA70.

Task 2. Study the effect of coactivators SRC-1, p300, and ARA70 on AR activation in the context of chromatin in Xenopus oocytes

Establishment of a hormone-responsive Xenopus oocyte transcription system

In order to gain insight into how AR regulates transcription in the context of chromatin, we chose to use the *Xenopus* oocyte as a model system. To express AR in *Xenopus* oocytes, oocytes were injected with *in vitro* synthesized mRNA encoding a Flag-tagged human AR and incubated overnight. Subsequent Western analysis using an AR-specific antibody revealed that injection of AR mRNA led to expression of AR in *Xenopus* oocytes, whereas the non-injected control oocytes had no detectable AR (Fig. 2A).

To investigate transcriptional regulation by AR in chromatin, we utilized two reporter constructs. Our previous work demonstrated that chromatin structure is important for transcriptional regulation of the *Xenopus* TRβA promoter by TR (34). We thus generated a TRβA promoter based reporter (4·ARE-TRβA) by inserting 4 copies of a consensus ARE upstream of the TRβA transcriptional start site (Fig. 2B). Since the functional importance of chromatin structure in transcriptional regulation of the MMTV LTR by steroid hormone receptors has been well established (3), we also generated a MMTV-LTR based reporter (Fig. 2B). To assemble reporter DNA into repressive chromatin through the replication-coupled chromatin assembly pathway (2, 34), we injected both reporters in ssDNA form into the nucleus of *Xenopus* oocytes. After overnight incubation, the injected oocytes were collected and the chromatin structure was analyzed by micrococcal nuclease (MNase) digestion assay. As shown in Fig. 2C, limited MNase digestions revealed that injection of both reporters as ssDNA plasmids led to the assembly of the DNA into chromatin with regularly spaced nucleosomes. This result is consistent with the notion that injection of ssDNA plasmid will result in efficient assembly of chromatin through a replication-coupled assembly pathway. It is noteworthy that our attempts to use a PSA enhancer-based reporter in Xenopus oocytes encountered with the problem of multiple transcription start sites, we thus restricted our studies to use the two reporters described above

We next examined whether expression of AR could activate transcription from repressive chromatin in *Xenopus* oocytes. Groups of *Xenopus* oocytes were injected with mRNA encoding AR (100 ng/μl, 18.3 nl/oocyte) and ssDNA of the MMTV reporter and treated with agonist

R1881 or the antagonists casodex or flutamide at concentrations ranging from 1 nM to 100 nM as indicated (Fig. 3). After overnight incubation, the total RNA was purified from each group of oocytes and the level of transcription from the MMTV promoter was analyzed by primer extension assay. A histone H4-specific primer, which detected the endogenous histone H4 mRNA and thus served as an internal loading control, was included in the primer extension reaction. As shown in Fig. 3, addition of R1881 at concentrations of 0.1 nM to 100 nM was sufficient to activate transcription from the MMTV promoter, whereas addition of casodex or flutamide failed to do so. Similar results were observed when the 4.ARE-TRβA reporter was used (data not shown). We thus conclude that AR expressed in *Xenopus* oocytes exhibits the expected hormone-specificity and activates transcription from the MMTV LTR assembled into chromatin.

AR possesses an intrinsic hormone-independent transcriptional activity

The above results demonstrate a hormone-dependent activation by AR from template in chromatin. An interesting result was observed when we next tested the effect of the levels of AR protein on transcriptional activation from both MMTV and TRβA-based reporters. We compared transcriptional activation from oocytes injected with a low dose (100 ng/μl) or a high dose (1 μg/μl) of AR mRNA and the reporter DNA (ssDNA) as indicated (Fig. 4A). Consistent with the result in Fig 3, a R1881-dependent activation was observed from both the MMTV and TRβA-based reporters when a low concentration of AR mRNA (100 ng/l) was injected (Fig. 4A). Injection of a high dose of AR mRNA (1 μg/μl) led to an even stronger R1881-dependent activation in comparison to injection of low dose of AR (compare lanes 5 with 3 and lanes 10 with 8). Importantly, injection of the high dose of AR mRNA also led to a R1881-independent activation of transcription from both reporters (compare lanes 4 with 2 and lanes 9 with 7). On the TRβA promoter, both R1881-independent and -dependent activation required the presence of AREs, because no activation was observed when the parental reporter without insertion of AREs was used as the reporter (data not shown), indicating that both R1881-dependent and independent activation are directly mediated by AR. Thus, not only have we observed R1881-stimulated transcription from both reporters as would be expected, but a ligand-independent trans-activation by AR was also observed when a high level of AR (see Fig. 3B) was expressed in *Xenopus* oocytes. Because ligand-independent activation for AR has only be reported in the cases of activation by cross-talk pathways and because the ligand-independent activity of AR has strong clinical implication in hormone-refractory prostate cancer, we decided to characterize further the molecular mechanism of this R1881-independent transcriptional activation by AR.

Overexpression of AR increased the level of AR in the nuclear fraction

Since several studies in mammalian cells have demonstrated that in the absence of ligand AR resides primarily in cytoplasm (14, 28), we first examined whether AR expressed in the *Xenopus* oocytes also exhibits a similar distribution. To do this, we took the advantage of the fact that the nucleus of *Xenopus* oocytes can be easily dissected manually away from the cytoplasm. Groups of *Xenopus* oocytes were injected with the low and high dose of AR mRNA as in Fig. 4A and incubated with or without addition of 10 nM of R1881. After overnight incubation, nuclear and cytoplasmic fractions were prepared from the oocytes and the distribution of AR was analyzed by Western blotting. As shown in Fig. 4B, in the absence of R1881, the majority of

AR was found in the cytoplasm in both groups of oocytes injected with the low and high doses of AR mRNA (compare lanes 1 and 2). Treatment with R1881 led to a strong enrichment of AR in the nuclear fraction (compare lanes 5 and 4). This result indicates that AR expressed in *Xenopus* oocytes is primarily localized to the cytoplasm in the absence of R1881 and undergoes translocation to the nucleus in response to R1881 treatment. Thus, the pattern of subcellular localization of AR proteins in *Xenopus* oocytes is identical to that in mammalian cells.

Importantly, as shown in Fig. 4B, overexpression of AR by injection of the high dose of AR mRNA (1 μg/μl) clearly increased the level of AR protein in the nucleus in the absence of R1881 (compare lane 2 in the Low and High). This result, together with the requirement of AREs for both R1881-dependent and -independent activation, suggests a model in which overexpression of AR leads to an increased level of AR protein in the nucleus and this nuclear AR leads to subsequent activation of transcription even in the absence of R1881.

DNA binding in vitro by AR protein is ligand-independent

The capacity of AR to activate transcription in the absence of hormone implies that AR can bind DNA in the absence of ligand. Since it is controversial as to whether ligand is required for DNA binding by AR, we analyzed the DNA binding activity of AR proteins expressed in *Xenopus* oocytes. We first carried out gel mobility shift assays using a ^{32}P-labeled ARE-containing oligonucleotide probe and oocyte extracts prepared from oocytes injected with AR mRNA and treated with or without R1881 (10 nM). To maintain the association with R1881 of the AR derived from the R1881-treated AR-expressing oocytes, a final concentration of 10 nM of R1881 was added to all buffers used for binding assay or for making extracts derived from the R1881-treated oocytes. As shown in Fig. 5, a shifted DNA complex can be observed in lanes with both AR programmed extracts, with (lane 3) or without R1881 (lanes 8), but not in the lanes with control oocyte extract (lanes 2 and 7). In addition, this complex is ARE-specific, since the complex could be eliminated by addition of an excessive cold ARE competitor but not cold TRE competitor. Furthermore, in multiple experiments, we observed that the AR-DNA complex in the presence of R1881 appeared to migrate slightly slower than that in the absence of R1881 (compare lane 3 with lane 8 and use the non-specific complex indicated by " * " as a reference). We suggest this difference in mobility may reflect the conformational changes of AR or/and the AR-DNA complex after binding of R1881.

To ensure that the AR indeed binds to the ARE in a sequence-specific manner, we also carried out DNase I footprinting assays. For this purpose, a ^{32}P-labeled DNA fragment from the TRβA promoter containing a single ARE insertion was generated by PCR and used as probe. AR expressed in oocytes treated with or without R1881 was partially affinity-purified using the Flag-tag specific M2 agarose beads to reduce the non-specific DNA binding by oocyte extracts. As shown in Fig. 6, AR purified from both R1881 untreated or treated oocytes can bind to the ARE in a dose-dependent manner. No significant difference can be observed in terms of the binding (or protection) of the ARE sequence by both R1881 treated or untreated AR. Interestingly, the protection by R1881-treated AR appeared to extend more broadly than that by unliganded AR (compare lane 7 with 4). This difference may reflect the difference in conformations between liganded and unliganded AR and/or association of liganded AR with additional protein(s). Taken together, both gel mobility shift and DNase I footprinting assays demonstrate convincingly that

AR binds to a consensus ARE in a ligand-independent manner, providing a crucial support for the observation of the ligand-independent activation.

Coactivators stimulate both R1881dependent and -independent activation

Since the activity of the NRs is subject to regulation by coactivators, we next tested the effect of SRC family coactivators and p300 on both hormone-dependent and independent activation by AR. We have yet to analyze the effect of ARA70 since we have not been able to express ARA70 in Xenopus oocytes so far. To better observe the effect of coactivators on ligand-independent activity of AR, we chose to express a moderate level of AR by injecting a medium concentration of AR mRNA (300 ng/μl). The expression of coactivators SRC-1, RAC3 or p300 was achieved by injection of their corresponding *in vitro* synthesized mRNA and confirmed by Western analysis [data not shown, see reference (20)]. As shown in Fig. 7A where the MMTV reporter was used, co-expression of SRC-1 and RAC3 with AR led to a significant enhancement of R1881-independent activation (from 4 fold to 16 and 17 fold, respectively). Under the same conditions, SRC-1 and RAC3 only moderately stimulated the transcription in the presence of R1881 (from 23 fold to 41 and 31 fold, respectively). The stimulation of R1881-independent activity by coactivators was not restricted to the MMTV reporter, as expression of p300 also stimulated the R1881-independent activation from the 4·ARE·TRβA based reporter from 7 to 22 fold (Fig. 7B). As a control, expression of p300 alone in the absence of AR did not stimulate transcription (Fig. 7B, compare lane 2 with 1), indicating that the stimulation of transcription by p300 is mediated through AR. Thus, much like the hormone-dependent activation, the hormone-independent activation can be enhanced by the action of coactivators such as SRC1, RAC3 and p300.

Ligand-independent activation by AR is also present in mammalian cells

To ascertain whether this ligand-independent activity by AR was unique to *Xenopus* oocytes, we also tested the ligand-independent activity of AR in mammalian cells by transient transfection. A luciferase reporter under the control of MMTV LTR was co-transfected with different amounts of an AR expression construct into COS-1 cells and treated with or without 10 nM of R1881. After 24 hours incubation, cells were collected and processed for the luciferase assay. As shown in Fig. 8, although R1881-independent activation by AR was not detectable when a low level of AR plasmid (5 ng) was used, the R1881-independent activation was clearly observed when a higher dose of AR expression plasmid (50 ng) was used. In both cases, addition of R1881 further stimulated the luciferase activity 4 and 3 folds, respectively. Thus, the ligand-independent activity by AR is not unique to *Xenopus* oocytes but likely an inherent feature of AR.

Task 3. Perform the structural and functional analysis of coactivators in Xenopus oocytes

We have focused our studies on coactivators p300 and SRC-1. We have generated a series of p300 mutants previously, including a mutant with severely reduced histone acetyltransferase activity (p300HATm) (Fig. 1). Transcriptional analysis in Xenopus oocytes indicated that, in comparison to the wild-type p300, this mutants was severely impaired in their ability to stimulate R-1881 stimulated activation by AR using MMTV reporter (Fig. 9). Similar

results were obtained when the TRβA reporter was used (data not shown). These results indicate that remodeling chromatin through histone acetylation is an essential step for transcriptional activation from chromatin.

In collaboration with Dr. Bert W. O'Malley, we have also generated a series of SRC-1 mutants (data not shown). We have verified the expression of this set of mutants in *Xenopus* oocytes after injection of their corresponding in vitro synthesized mRNA by Western blotting. We are currently determining the structural and functional domains of the SRC-1 important for AR activation using this set of mutants.

Task 4. Analyze the protein-protein interaction between coactivators

We have analyzed the protein-protein interaction between SRC family coactivators and p300. First, we confirmed by co-immunoprecipitation experiments the interaction between the SRC family coactivators and p300. Second, by using the p300ΔSRC mutant described above, we demonstrated that the interaction required the SRC interaction domain in p300. These results were published last year (20) and thus we will not show data here. Recently, we have also confirmed that the p300 interaction domain in SRC-1 is also important for the interaction. We have yet to analyze the interaction of ARA70 with SRC family coactivators and p300, if any, since we have not been able to express ARA70 in Xenopus oocytes so far. However, preliminary study using transient transfection has failed to show any significant interaction of ARA70 with SRC-1 and p300, suggesting that ARA70 may not interact with both of them.

Task 5. Purification of functionally active p300 protein from SF9 cells

We obtained p300 expression baculovirus from Dr. Lee Karus at Cornell University. By using a purification scheme as described, we affinity purified the His6-tagged p300 from baculovirus infected SF9 cells by using Ni-NTA agarose chromatography. As shown in Figure 10, the purified p300 was at least 90% homologous based on the Coomassie blue staining after SDS-PAGE. Furthermore, such purified p300 proteins contained a potent HAT activity as revealed by HAT assay using core histones as substrate (data not shown). Unfortunately, Storm Allison caused the loss of power to the storage freezer for more than three days and all our p300 preparations were inactivated. We now just have to prepare again the p300 proteins.

Task 6. Preparation of functionally active ARA70 from injected Xenopus oocytes

No progress was made in this task since we could not express ARA70 in Xenopus oocytes so far. We currently are trying the alternative approach using baculovirus expression system to express and prepare ARA70. We hope this alternative approach will allow us to study the function of ARA70 in AR activation in vitro in near future.

Task 7-12 (see original proposal)

All those tasks were proposed as work after first year. We already have some progress in those areas too. We are currently in the process of establishing a chromatin based in vitro transcription system for AR. Unfortunately, Storm Allison caused big setback of this research.

As mentioned earlier, our precious p300 preparation lost activity due to this storm. Furthermore, our precious preparation of Drosophila S-190 embryo extracts also lost activity during the storm. Preparation of these extracts were time-consuming and were done as collaboration in Dr. Carl Wu'laboratory at NCI/NIH. We are now in the painful process to prepare p300 and Drosophila S-190 embryo extracts all over again. Together with the loss of p300 protein preparation (task 5), we believe Storm Allison resulted in significant setback on our research progress. We had submitted a letter petitioned for a half year extension of the support due to the loss caused by Storm Allison (see appendix 2).

Regarding to the proposed work on the mutant AR(877Thr-Ala) found frequently in prostate cancer patients, we have generated this mutant AR in the laboratory by site-directed mutagenesis (data not shown). We are currently on the process to characterize and compare this mutant AR and wild-type AR in their ability to activate transcription and response to agonists and antagonists.

Statement of work accomplished/in progress

Task 1. Generate the constructs for and analyze the expression of p300 and ARA70 in Xenopus oocytes. **Accomplished except problem for ARA70.**

Task 2. Study the effect of coactivators SRC-1, p300, and ARA70 on AR activation in the context of chromatin in Xenopus oocytes. **Accomplished except problem for ARA70.**

Task 3. Perform the structural and functional analysis of coactivators in Xenopus oocytes. **Accomplished for p300 and significant progress for SRC-1.**

Task 4. Analyze the protein-protein interaction between coactivators. **Accomplished for SRC-1 and p300.**

Task 5. Purification of functionally active p300 protein from SF9 cells. **Accomplished.**

Task 6. Preparation of functionally active ARA70 from injected Xenopus oocytes. **In progress due to problem of expression in Xenopus oocytes. Initiating a new approach (baculovirus) for expression of ARA70.**

Task 7-12. In progress.

Key Research Accomplishments

- Expression constructs for SRC-1 and p300 have been generated.
- Expression of SRC-1 and p300 in Xenopus oocytes was verified.
- A R-1881 responsive AR-dependent transcription activation from Xenopus oocytes were established.
- A hormone-independent activation was observed when AR was expressed in high levels in both Xenopus oocytes and mammalian cells.

- A hormone-independent DNA binding activity of AR proteins expressed in Xenopus oocytes was demonstrated.
- A variety of mutants for p300 and SRC-1 were generated.
- Structural and functional analyses revealed that both SRC interaction and HAT activity were required for p300 to facilitate AR activation.
- Coactivators SRC-1 and p300 stimulate both hormone-dependent and independent activation by AR.
- Functionally active p300 proteins were prepared using a baculovirus expression system.

Reportable Outcomes

A manuscript reporting the observation that AR contains an intrinsic hormone independent transcriptional activity is in preparation. In addition, as collaboration with Dr. Yi Zhang at University of North Carolina, we have recently reported in Science (see appendix 2) that the coactivator PRMT1 is a histone methyltransferase that specifically methylates the arginine 3 of the histone 4 (31). Expression of PRMT1 in Xenopus oocytes stimulates AR activity and this coactivator activity requires its histone methyltransferase activity. Although this work was not progressed in the original proposal, we tribute this work to the support of DOD-.

Conclusions

Significant progress has been made in most of the tasks proposed for the first years. We have established an AR-dependent, hormone-responsive transcription system using Xenopus oocytes. This has allowed us to demonstrate that coactivators SRC-1 and p300 stimulate AR activation in the context of chromatin. In so doing, p300 requires its interaction with SRC-1 and its intrinsic HAT activity. Furthermore, we observe that AR contains also an intrinsic hormone-independent trans-activation activity. This activity is observed when a high level of expression of AR is achieved. This activity is observed in both Xenopus oocytes and mammalian cells. We believe this hormone-independent activity of AR may be relevant to the possible function of AR in hormone-independent prostate cancer. Finally, we demonstrate that histone methylation is also likely to have an important role in transcriptional regulation by AR, as the coactivator PRMT1 is found to be a histone H4 Arg-3 specific methyltransferase (see appendix 3). Taken together, we believe the establishment of a hormone-responsive chromatin-based transcription system using Xenopus oocytes will allow us to further elucidate the molecular mechanisms by which AR regulates transcription in the context of chromatin and by which AR retains its trans-activation function in hormone-independent prostate.

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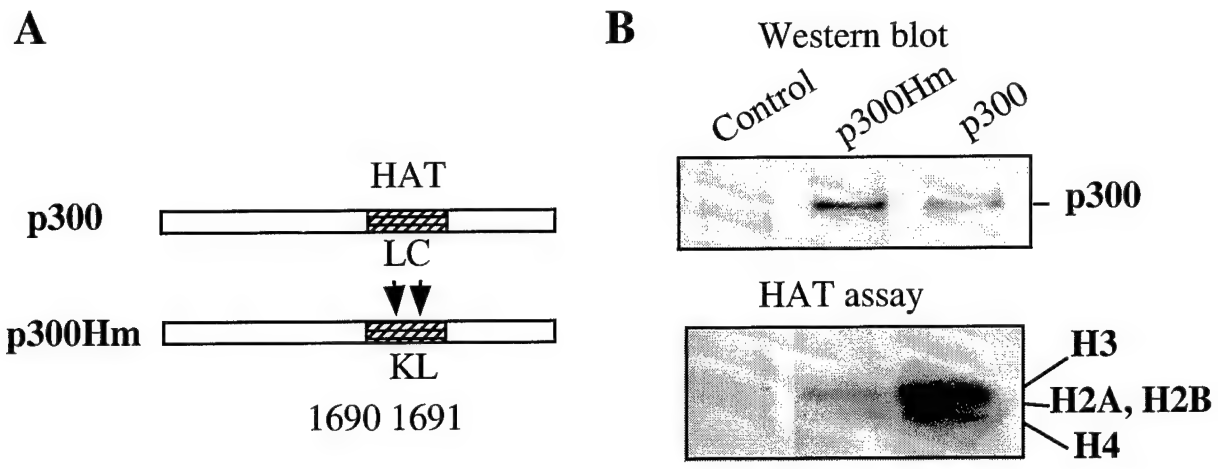

Fig. 1. Expression of p300 and its HAT mutant in Xenopus oocytes. (A) Schematic presentation of the p300 and p300Hm, in which leucine 1690 is converted to lysine and cysteine 1691 is converted to leucine. (B). Western blot using a p300 specific antibody showing the expression of p300 and the p300Hm in Xenopus oocytes after injection of their corresponding in vitro synthesized mRNA. Both p300 and p300Hm expressed in Xenopus oocytes were then immunoprecipitated using the p300 specific antibody and the HAT activity was assayed in vitro using core histones as substrate.

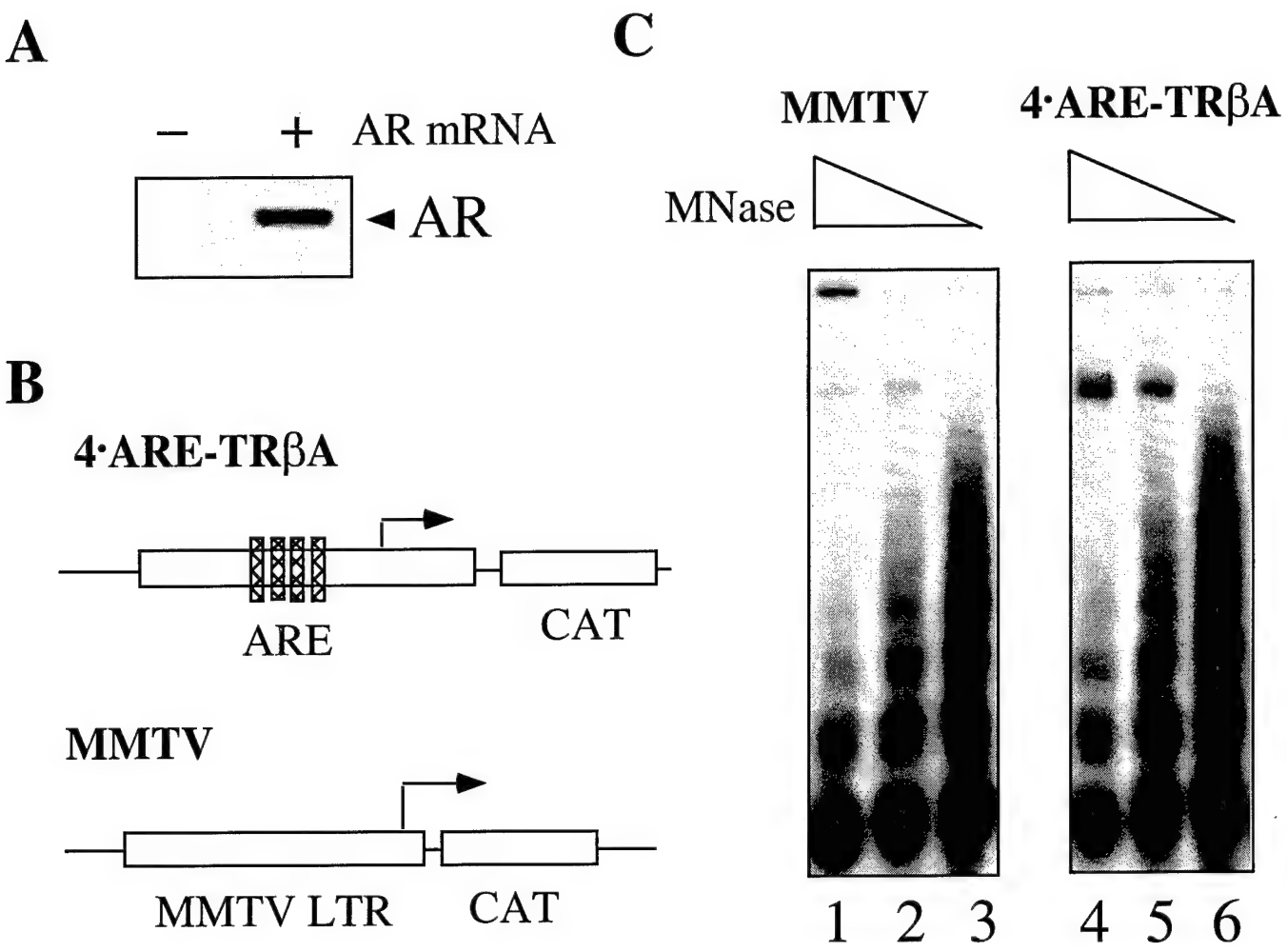

Fig. 2. Expression of AR and assembly of AR-responsive reporters into chromatin in *Xenopus* oocytes through microinjection. A). Western analysis using an AR specific antibody of extracts derived from control oocytes (-) and oocytes injected with AR mRNA (+) (100 ng/μl, 18.4 nl/oocyte). B). Diagram showing the structure of 4.ARE-TRβA promoter based and MMTV LTR based reporters. The arrow indicates the transcriptional start site. C). Both reporters were assembled into chromatin with regularly spaced nucleosomal arrays via replication-coupled pathway. The ssDNA of both reporters was injected into the nucleus of *Xenopus* oocytes (50 ng/μl, 18.4 nl/oocyte). After overnight incubation, the chromatin structure was analyzed by MNase assay.

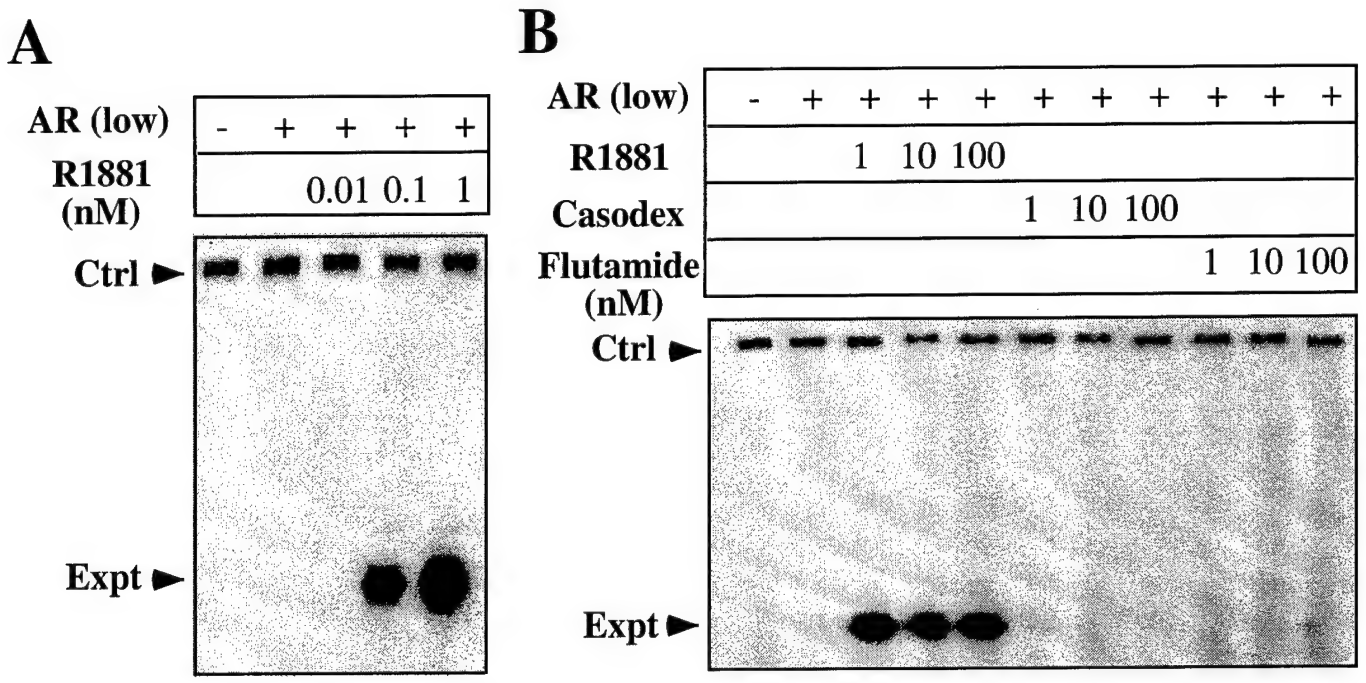

Fig. 3. R1881 but not the antagonists casodex and flutamide stimulates AR transcriptional activation. Groups of oocytes were injected with a low dose of AR mRNA (100 ng/μl, 18.4 nl/oocyte) and ssDNA of MMTV reporter (50 ng/μl, 18.4 nl/oocyte). The oocytes were then treated overnight with R1881 or the antagonists casodex or flutamide at a concentration as indicated. The levels of transcription were then analyzed by primer extension assay. Ctrl: the primer extension product derived from the endogenous storage histone H4 mRNA. Expt: the primer extension product derived from transcripts from the MMTV LTR reporter.

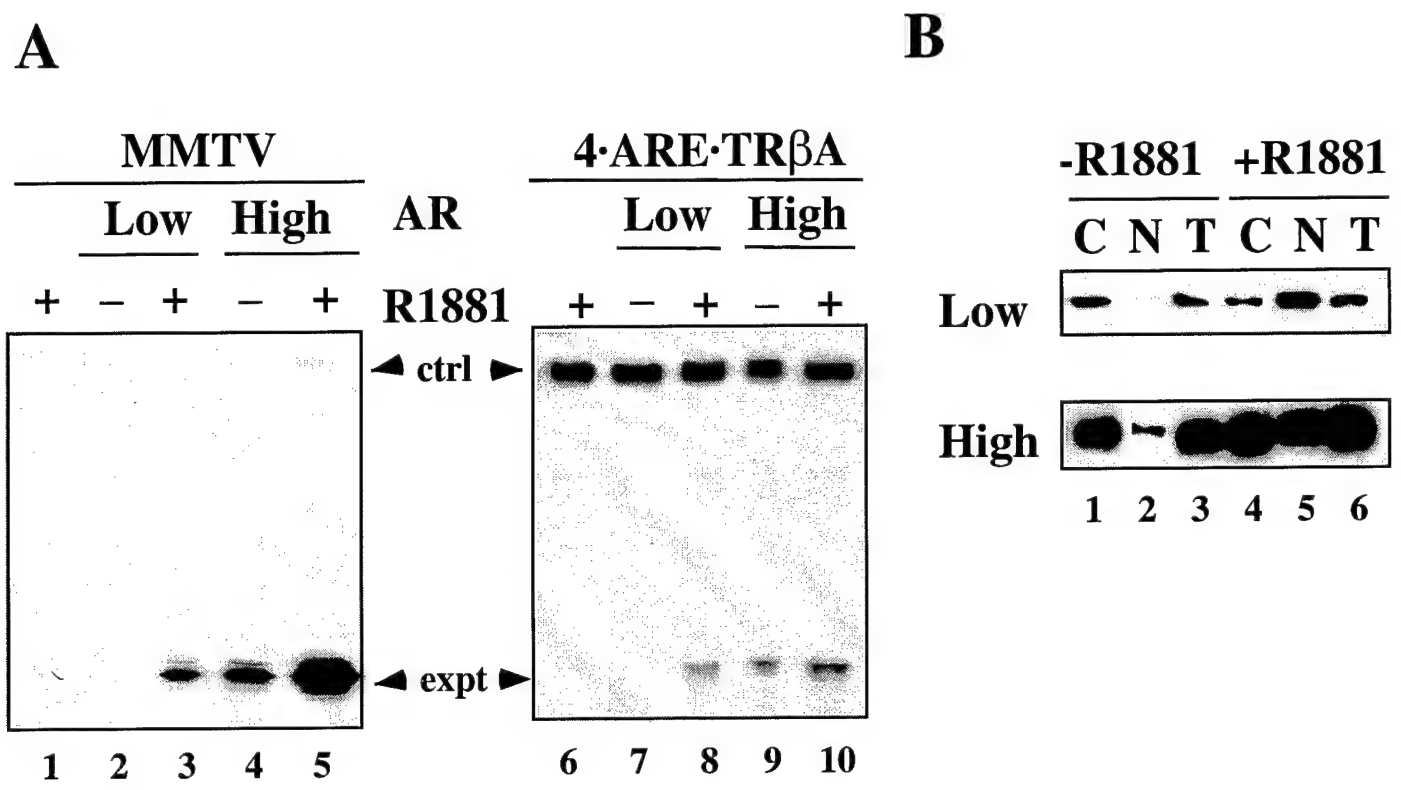

Fig. 4. AR exhibits both R1881-dependent and -independent activation. A). Injection of low and high doses of AR mRNA led to observation of both R1881-dependent and -independent activation. Groups of oocytes were injected with AR mRNA at low (100 ng/μl, 18.4 nl/oocyte) or high concentrations (1 μg/μl, 18.4 nl/oocyte) and treated with or without R1881 (10 nM) overnight. Both reporters were injected as ssDNA as in Figure 1C. The primer extension assay was as in Fig. 2. B). Subcellular localization of AR expressed in *Xenopus* oocytes. Groups of oocytes were injected with AR mRNA at low (100 ng/μl, 18.4 nl/oocyte) or high concentrations (1 μg/μl, 18.4 nl/oocyte) and treated with or without R1881 (10 nM) overnight as in A). The nuclear (N) and cytoplasmic (C) fractions of the oocytes were then dissected manually and analyzed for AR proteins by Western blotting using a Flag-tag specific antibody (M2, Sigma). T: total oocyte extract.

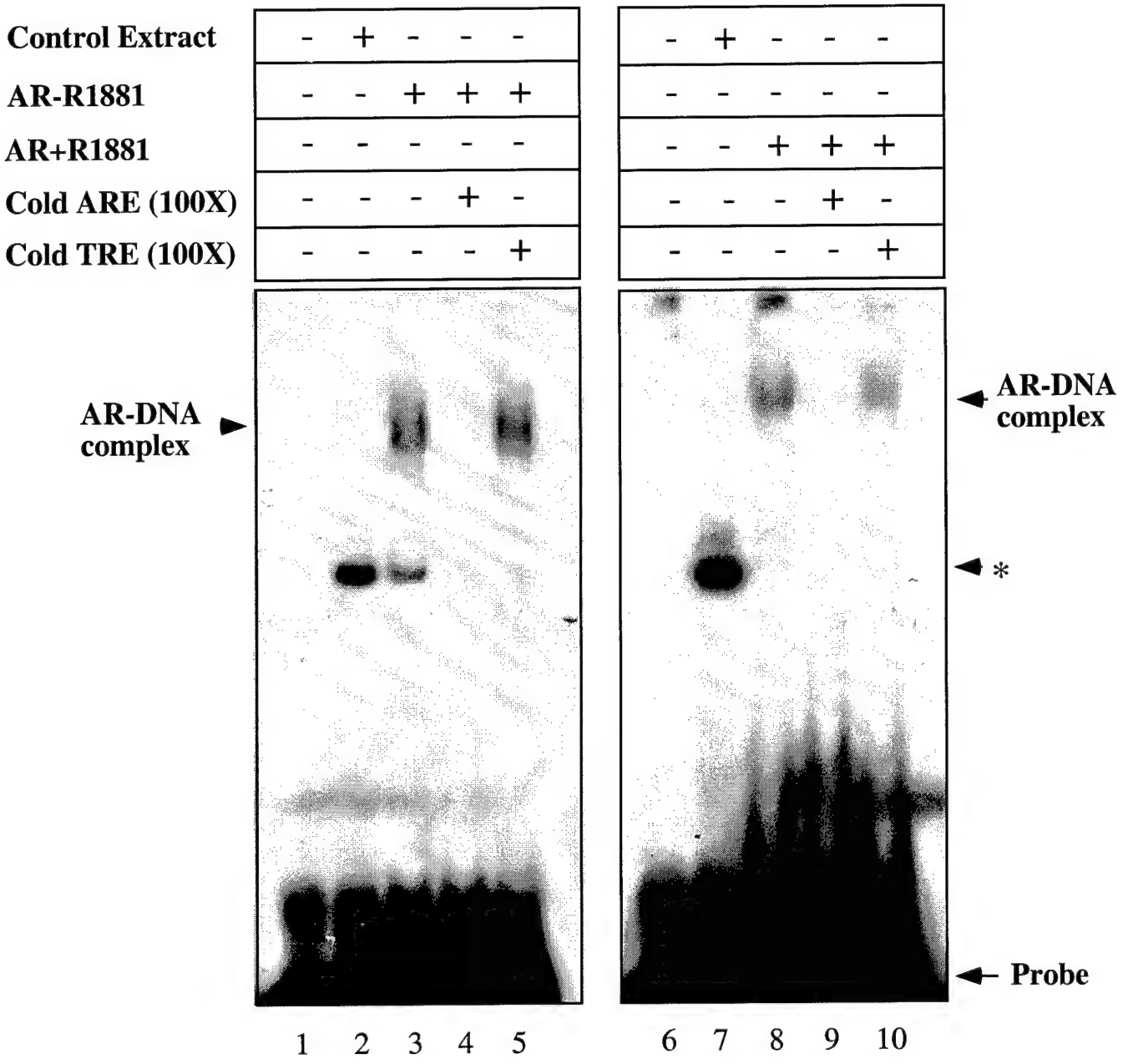

Fig. 5. AR expressed in *Xenopus* oocytes binds to a consensus ARE in a ligand-independent manner. The extracts prepared from control oocytes or oocytes injected with AR mRNA (1 μg/μl, 18.4 nl/oocyte) and treated with or without R1881 (10 nM) were used for gel mobility shift assays. The "*" indicates a non-specific protein-DNA complex also present in the control oocytes. This non-specific complex can be competed by addition of both ARE and TRE competitors. The position of AR-DNA complex is also indicated. Note that the AR-DNA complex is present only in the AR-containing extracts and can be competed out by addition of an excessive cold ARE but not the TRE competitor.

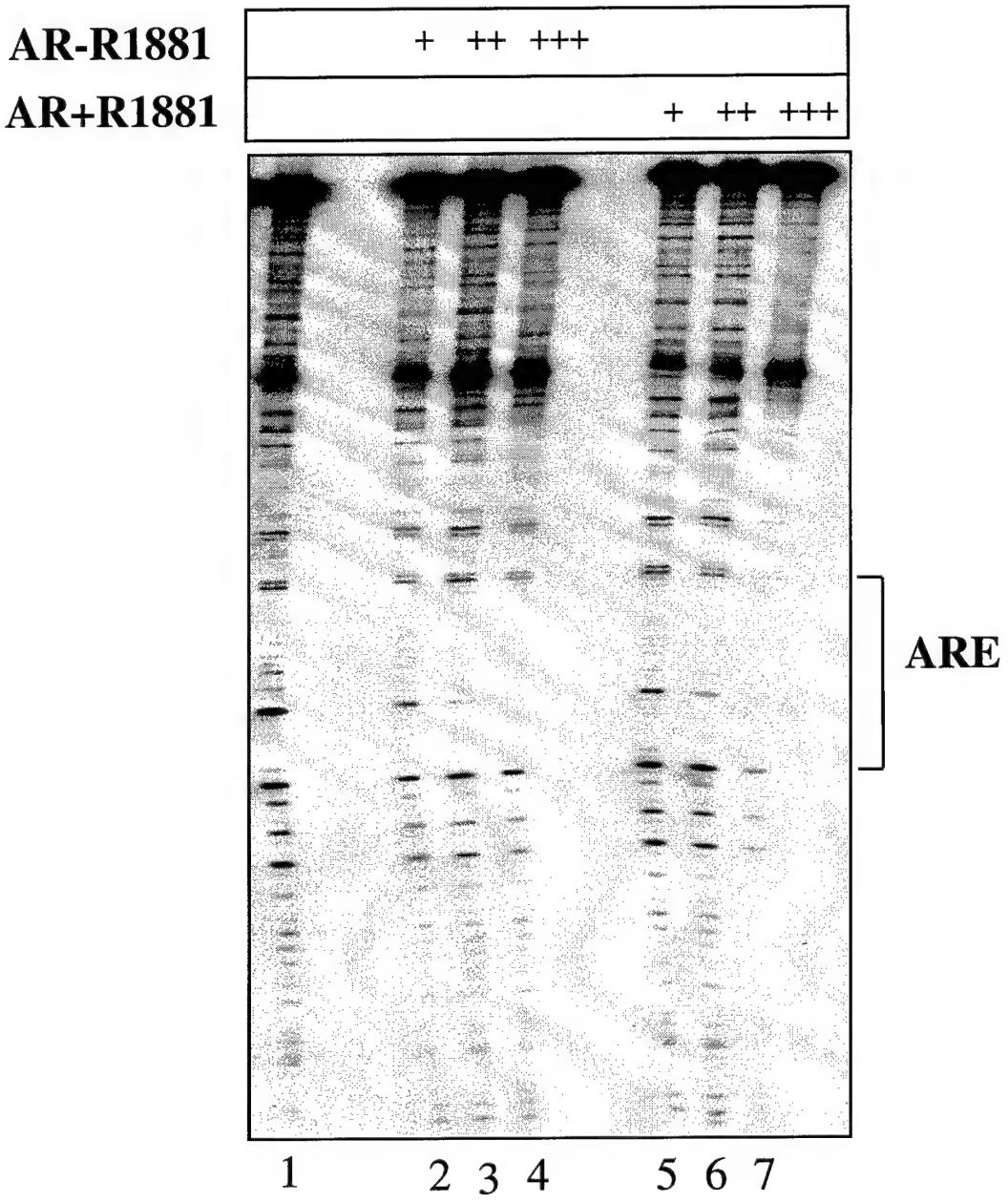

Fig. 6. DNase I footprinting assays indicate that AR binds in a ligand-independent manner specifically to the ARE. The end-labeled probe containing a consensus ARE sequence was generated by PCR. An increasing amount of partially purified AR (2 µl in lanes 2 and 5, 4 µl in lanes 3 and 6, and 8 µl in lanes 4 and 7) were used in the DNase I foot-printing assay. The position of the ARE is as indicated.

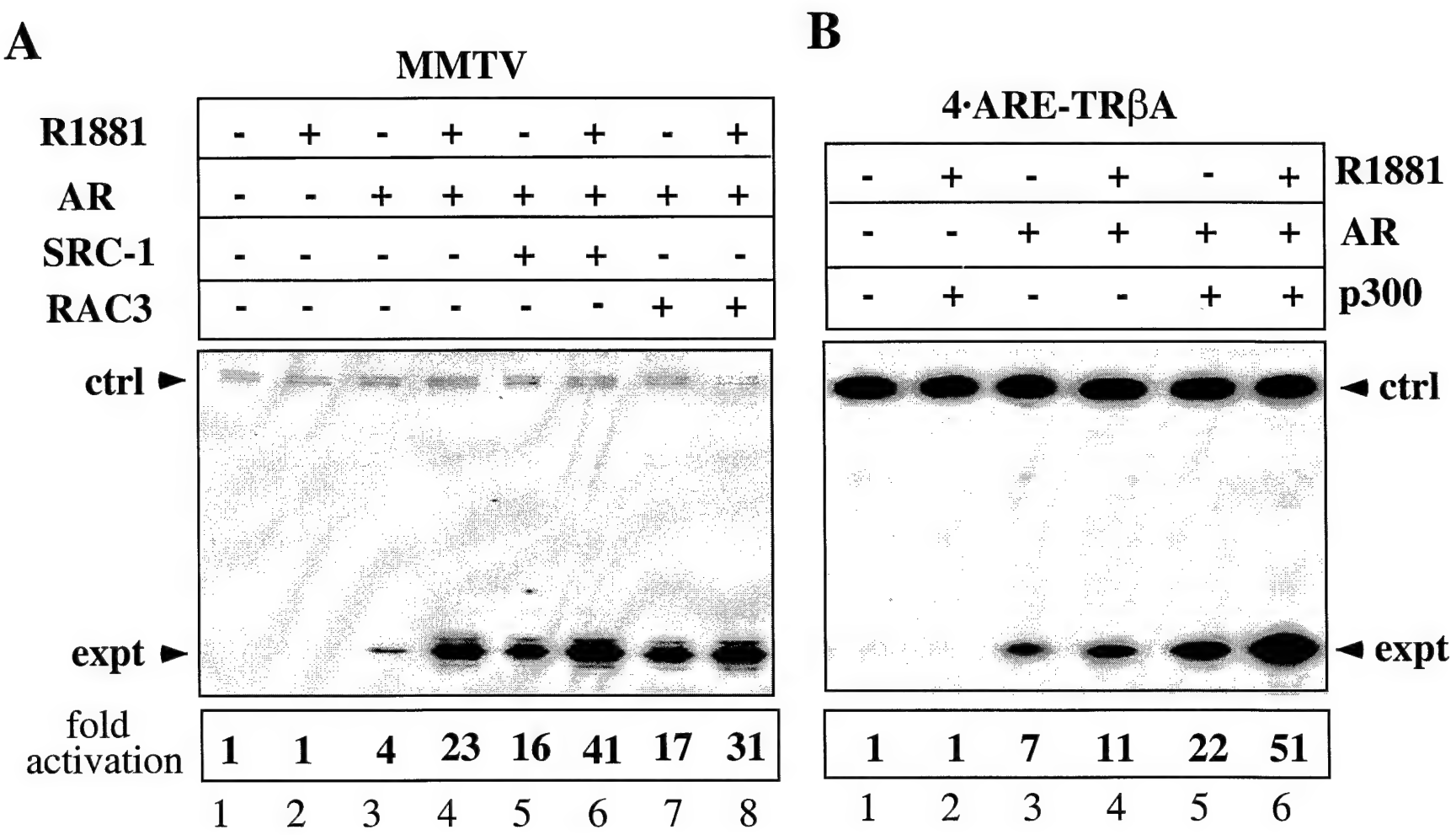

Fig. 7. Coactivators such as SRC-1, RAC3 and p300 stimulate both ligand-dependent and independent activation by AR. A). Both SRC-1 and RAC3 enhanced R1881-dependent and independent activation by AR. Groups of oocytes were injected with a medium concentration of AR mRNA (300 ng/μl, 18.4 nl/oocyte) and mRNA encoding SRC-1 or RAC3 (100 ng/μl, 18.4 nl/oocyte) as indicated. The oocytes were then injected with ssDNA of the MMTV reporter and treated with or without R1881 (10 nM) overnight. The primer extension assay was as in Fig. 2. B). The coactivator p300 stimulates both R1881-dependent and independent activation. The experiment was as in A) except that mRNA encoding p300 and the 4.ARE-TRβA reporter were used.

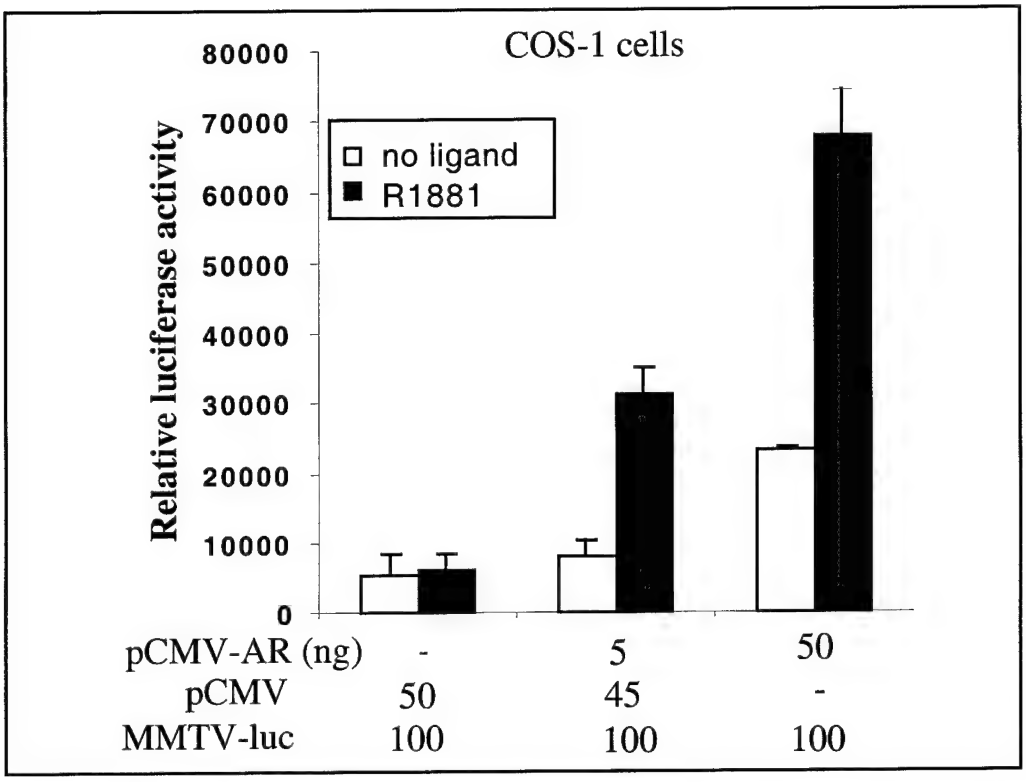

Fig. 8. Hormone-independent activation by AR is also observed in mammalian cells. COS-1 cells were transiently transfected with MMTV-LTR-luc reporter and expression vector for AR as indicated. The luciferase data, expressed as relative light units, are the mean and standard deviation of three independent transfection experiments

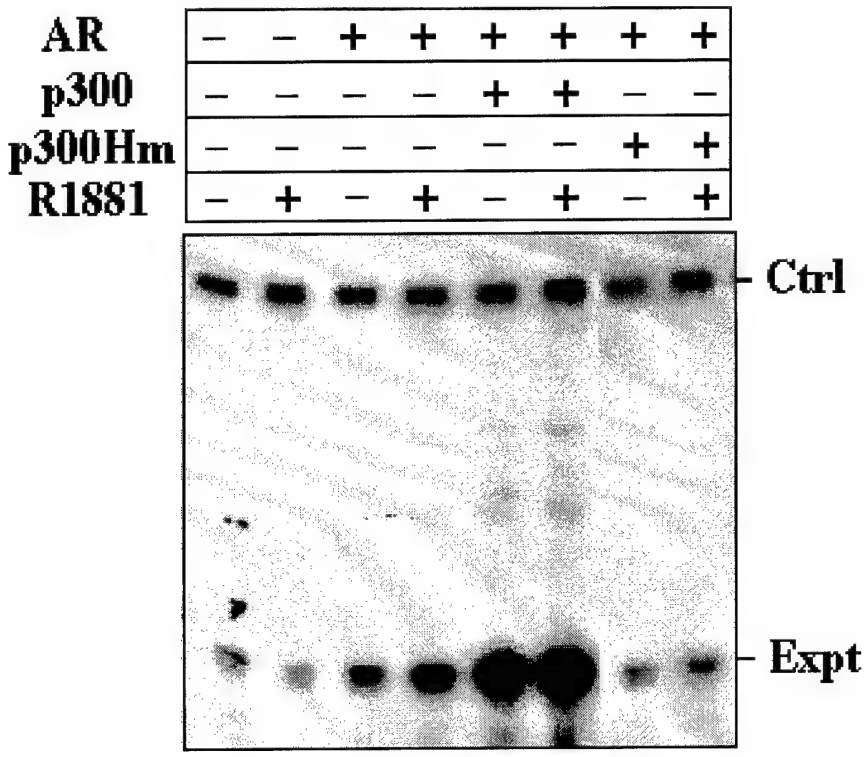

Fig. 9. The HAT activity of p300 is essential for its ability to stimulate AR activation in Xenopus oocytes. Groups of oocytes were injected with AR mRNA (100 ng/μl) and p300 or p300Hm as indicated. The reporter DNA used is MMTV-CAT. R1881: 10 nM.

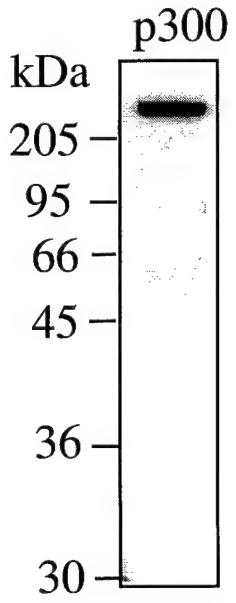

Fig. 10. Preparation of p300 proteins using a baculovirus expression system. The full-length 6xHis-tagged p300 was purified from SF9 cells using Ni-NTA agarose chromatography and analyzed by a SDS-PAGE followed by Coomassie blue staining. All indicated are molecular weight markers.

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July 30, 2001

Patricia C. Modrow, Ph.D.
CDMRP Deputy Director for Grants Management

Dear Dr. Modrow,

First of all, I will like to express my gratitude for your concern and empathy on our flood damage from the Tropical Storm Allison. Here I summarized several areas of our research supported by DOD that were affected by this storm:

1. The most devastating effect was on our task to characterize coactivators *in vitro* (Task 5 and 6). Most of our precious purified recombinant p300 and SRC family coactivator proteins were lost due to the lack of power. Freezers were thawed, even with our tremendous effort to put dry ice into the freezers. It would require at least 3 months to express and re–purify of those lost proteins.

2. Our Drosophila embryo extracts crucial for chromatin assembly in vitro were also affected (Task 7, 8 and 11). The assembly activity was much reduced after this storm. These extracts were generated at Dr. Carl Wu's laboratory at NCI/NIH as a collaboration. We now have to make all extracts for chromatin assembly again. The preparation of those extracts is not only time-consuming, it also requires us to travel and stay at NIH for at least a week.

3. Many of the common enzymes (restriction enzymes and others) were affected by this storm.

Fortunately we did not lose any major equipment to the storm. However, as a research scientist, the most precious lost was time. Adding up all those proteins and extracts we have to make again together with the disruption on other experiments, we estimate that this storm would delay our research at least about half a year. I thus like to request for half a year extension of our performance period. I thank you very much for your kind consideration.

Sincerely Yours,

Jiemin Wong, Ph.D.

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particularly in lizards and birds, which commonly have derived nasal vestibules. The dearth of exceptions in the face of this diversity emphasizes the fundamental nature of the relationship.
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18. Why did anyone ever place the nostril caudally in dinosaurs? All leading early paleontologists regarded the largest dinosaurs (sauropods) as primarily "amphibious" (*32–35*). This view was clinched by the discovery in the sauropod *Diplodocus* of a large caudodorsal nasal opening (*36*) that was thought to serve as a snorkel, allowing the nearly submerged dinosaur to breathe (the dorsal opening in *Diplodocus* is actually just the caudal portion of the bony nostril, and the rostral portion extends far forward as a shallow narial fossa; vascular relationships confirm a rostral nostril). Although sauropods were later interpreted as terrestrial (*37*), the inferred caudal nostril stuck and somehow was transferred to other dinosaurs. Retracted nasal bones perhaps could be evidence, but nasal retraction is common in mammals in association with development of a proboscis, and mammals have been studied as extant analogs [e.g., (*1*, *38*)]. Despite retraction of the bony nostril, the fleshy nostril remains rostrally positioned; moreover, some mammals with retracted nasals (e.g., tapirs, elephants) enhance the fundamental amniote rostroventral position by extending the fleshy nostril out on a trunk. Thus, caudal expansion of the bony nostril apparently is not driven by caudal movement of the fleshy nostril, but rather by caudal expansion of the vestibular contents, that is, the narial apparatus.
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18 May 2001; accepted 27 June 2001

Methylation of Histone H4 at Arginine 3 Facilitating Transcriptional Activation by Nuclear Hormone Receptor

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Acetylation of core histone tails plays a fundamental role in transcription regulation. In addition to acetylation, other posttranslational modifications, such as phosphorylation and methylation, occur in core histone tails. Here, we report the purification, molecular identification, and functional characterization of a histone H4–specific methyltransferase PRMT1, a protein arginine methyltransferase. PRMT1 specifically methylates arginine 3 (Arg 3) of H4 in vitro and in vivo. Methylation of Arg 3 by PRMT1 facilitates subsequent acetylation of H4 tails by p300. However, acetylation of H4 inhibits its methylation by PRMT1. Most important, a mutation in the *S*-adenosyl-L-methionine–binding site of PRMT1 substantially crippled its nuclear receptor coactivator activity. Our finding reveals Arg 3 of H4 as a novel methylation site by PRMT1 and indicates that Arg 3 methylation plays an important role in transcriptional regulation.

Covalent modifications of core histone tails play important roles in chromatin function (*1*). One type of covalent histone modification is methylation (*2*), which has been observed in diverse organisms from yeast to human (*3*). However, the consequence of this posttranslational modification is not understood. One major obstacle in understanding the function of histone methylation is the lack of information about the responsible enzymes. The demonstrations that SUV39H1, the human homolog of the *Drosophila* heterochromatic protein Su(var)3-9, is an H3-specific methyltransferase (*4*) and that methylation of lysine 9 (Lys 9) on histone H3 serves as a binding site for the heterochromatin protein 1 (HP1) (*5–7*) underscore the importance of histone lysine methylation in heterochromatin function. Methylation of histones can occur on arginine residues, as well as lysine residues (*8*). The recent demonstrations that a nuclear receptor coactivator-associated protein, CARM1, is an H3-specific arginine methyltransferase suggests that histone arginine methylation may be involved in transcriptional activation (*9*).

To identify enzymes involved in core histone methylation, nuclear proteins from HeLa cells were separated into nuclear extract and nuclear pellet followed by further fractionation on DEAE52 and phosphate cellulose P11 columns. Fractions derived as above were assayed for methyltransferase activity by using core histone octamers as substrates (*10*). Multiple methyltransferase activities with distinctive specificity for histones H3 and H4 were seen (Fig. 1A). By following histone methyltransferase (HMT) activity (Fig. 1A), we purified an H4-specific HMT from the nuclear pellet fraction to homogeneity (*11*). Analysis of the column fractions derived from the hydroxyapatite column indicated that the peak of the enzymatic activity eluted in fraction 14 and trailed through fraction 26 (Fig. 1B, bottom panel). Silver staining of an SDS-polyacrylamide gel containing the column

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fractions revealed that a polypeptide of 42 kD coeluted with the enzymatic activity (Fig. 1B, top panel). To confirm this result, the same input was loaded onto a gel-filtration Superose-200 column. Analysis of the column fractions indicated that the peak of the enzymatic activity eluted around 330 kD between fractions 38 and 41 (Fig. 1C, bottom panel). Silver staining of an SDS-polyacrylamide gel containing the column fractions revealed again that a 42-kD polypeptide coeluted with the enzymatic activity. Mass spectrometry analysis (*11*) identified the 42-kD polypeptide as the human protein arginine *N*-methyltransferase 1, PRMT1 (*12*). Because the HMT activity eluted around 330 kD and only coeluted with PRMT1, it is likely that PRMT1 functions as a homo-oligomer. This was verified by the demonstration that recombinant PRMT1 fractionated in the same way as the endogenous PRMT1, as a 330-kD complex (*11*). Therefore, we conclude that PRMT1 functions as an H4-specific HMT in the form of a homo-oligomer.

The identification of PRMT1 as one of the most abundant H4-specific HMTs is surprising, because only Lys 20 of H4 has been reported to be methylated in vivo (*1*) and because PRMT1 is not known to be able to methylate lysine residues. Instead, PRMT1 and its yeast homolog have been reported to mainly methylate arginine of certain RNA binding proteins (*8*). To determine whether PRMT1 methylates H4 on Lys 20, core histone octamers were methylated with recombinant or native PRMT1 in the presence of *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]SAM). After separation by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), methylated H4 was recovered and mi-

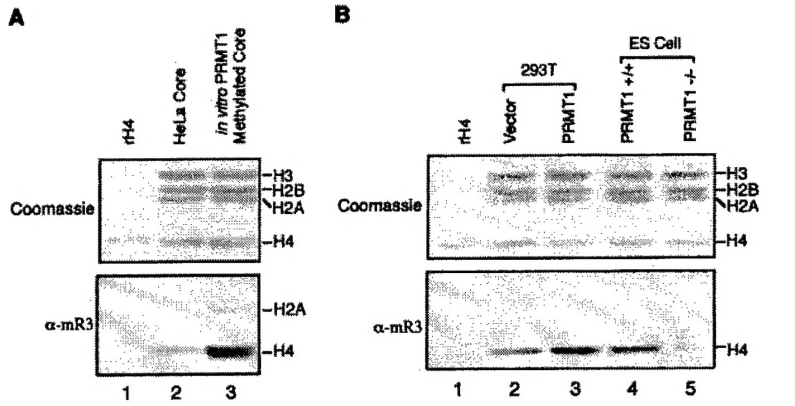

Fig. 2. PRMT1 methylates Arg 3 of H4 in vitro and in vivo. (**A**) Arg 3 methylation occurs in vivo. Recombinant histone H4 (200 ng) and equivalent amount of core histones from HeLa cells that were either subjected to mock (lane 2) or PRMT1 (lane 3) methylation before loading to SDS-polyacrylamide gel for Coomassie and Western blot analysis using the methyl-Arg 3–specific antibody. (**B**) PRMT1 is responsible for Arg 3 methylation in vivo. Recombinant histone H4 (200 ng) and equivalent amount of core histones purified from transiently transfected 293T cells (*23*) or ES cells were analyzed by Coomassie and Western blot as in (A).

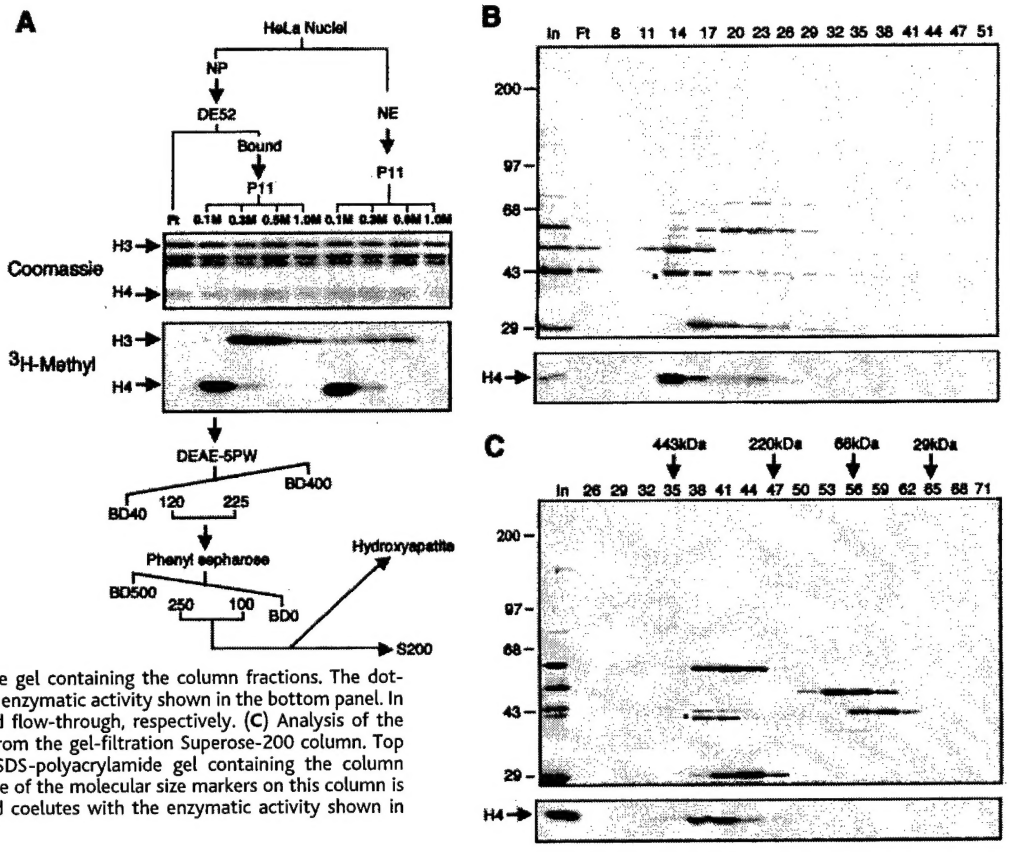

Fig. 1. Purification and molecular identification of an H4-specific methyltransferase. (**A**) HeLa nuclear proteins were fractionated as described (*11*) and fractions were assayed for histone methyltransferase activity (*10*). (Top) A Coomassie-stained gel indicating equal amounts of core histone substrate are used in each reaction. (Bottom) A fluorogram of the same gel indicating different fractions contain different HMT activities with specificity for H3 and/or H4. Equal volumes of the protein fractions were used in the enzymatic assay. Protein concentrations of the fractions from lanes 1 to 9 are 0.3, 0.3, 0.4, 0.4, 0.4, 1.2, 0.6, 0.3, and 1.2 mg/ml, respectively. (**B**) Analysis of the column fractions derived from the hydroxyapatite column. (Top) A silver-stained SDS-polyacrylamide gel containing the column fractions. The dotted-band coelutes with the enzymatic activity shown in the bottom panel. In and Ft represent input and flow-through, respectively. (**C**) Analysis of the column fractions derived from the gel-filtration Superose-200 column. Top panel is a silver-stained SDS-polyacrylamide gel containing the column fractions. The elution profile of the molecular size markers on this column is indicated. The dotted-band coelutes with the enzymatic activity shown in the bottom panel.

crosequenced by automated Edman chemical sequencing. Sequentially released amino acid derivatives were collected and counted by liquid scintillation, revealing that Arg 3, instead of Lys 20, was the major methylation site (*11*). Comparison of the ability of PRMT1 to methylate H4 tail peptides with or without a mutation on Lys 20 showed no difference, confirming that Lys 20 is not a site for PRMT1 methylation (*13*).

The identification of H4 Arg 3 as an in vitro methylation site for PRMT1 is intriguing. To determine whether Arg3 methylation occurs in vivo, antibodies against an Arg 3–methylated histone H4 NH_2-terminal peptide were generated (*14*). Although the antibody reacted strongly with PRMT1-methylated H4, it did not recognize equal amounts of recombinant H4 expressed in *Escherichia coli* (Fig. 2A, compare lanes 1 and 3), indicating that the antibody is methyl-Arg 3–specific. This same antibody also recognized histone H4 purified from HeLa cells (Fig. 2B, lane 2) indicating certain amount of Arg 3-methylation occurs in vivo. We note that H2A can also be weakly methylated by PRMT1 in vitro and that methylated H2A can be recognized by the methyl-Arg 3 antibody (Fig. 2A, compare lanes 2 and 3). The methylation site on H2A is likely to be Arg 3, because H2A has the same extreme NH_2-terminal sequence "SGRGK" as that of H4 (*14*). However, the amount of endogenous H2A methylation is undetectable under the same conditions (bottom panels of Fig. 2, A and B).

We next sought to determine whether PRMT1 is responsible for this site-specific Arg 3 methylation in vivo. If PRMT1 is responsible for Arg 3 methylation, overexpression of PRMT1 should increase the amount of Arg 3 methylation. The results shown in Fig. 2B indicate that overexpression of PRMT1 increases Arg 3 methylation (compare lanes 2 and 3). To confirm the above result, core histones from *PRMT1*$^{+/+}$ and *PRMT1*$^{-/-}$ embryonic stem (ES) cells (*15*) were purified and compared for their Arg 3 methylation. The results shown in Fig. 2B (compare lanes 4 and 5) demonstrated that inactivation of the *Prmt1* gene results in a dramatic decrease in the amount of Arg 3 methylation, indicating that histone H4 is likely an in vivo substrate for PRMT1. However, we could

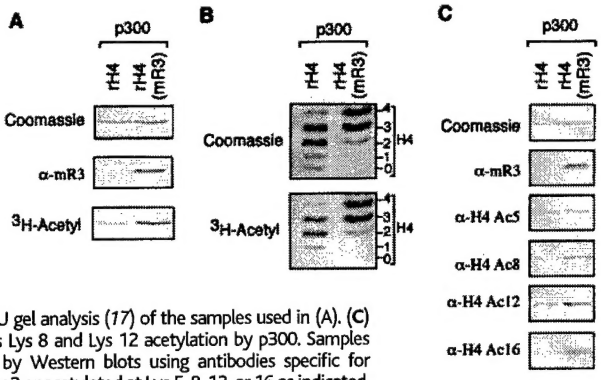

Fig. 3. Arg 3 methylation stimulates H4 acetylation by p300. (**A**) PRMT1-methylated H4 is a better substrate for p300 acetylation. Mock- and PRMT1-methylated recombinant H4 were subjected to p300 acetylation in the presence of [^{3}H]acetyl-CoA (*16*). Samples were analyzed by Coomassie, Western blot, and fluorogram. (**B**) TAU gel analysis (*17*) of the samples used in (A). (**C**) Arg 3 methylation facilitates Lys 8 and Lys 12 acetylation by p300. Samples used in (A) were analyzed by Western blots using antibodies specific for histone H4 methylated at Arg 3 or acetylated at Lys 5, 8, 12, or 16 as indicated. The site-specific acetyl-lysine antibodies are purchased from Serotec.

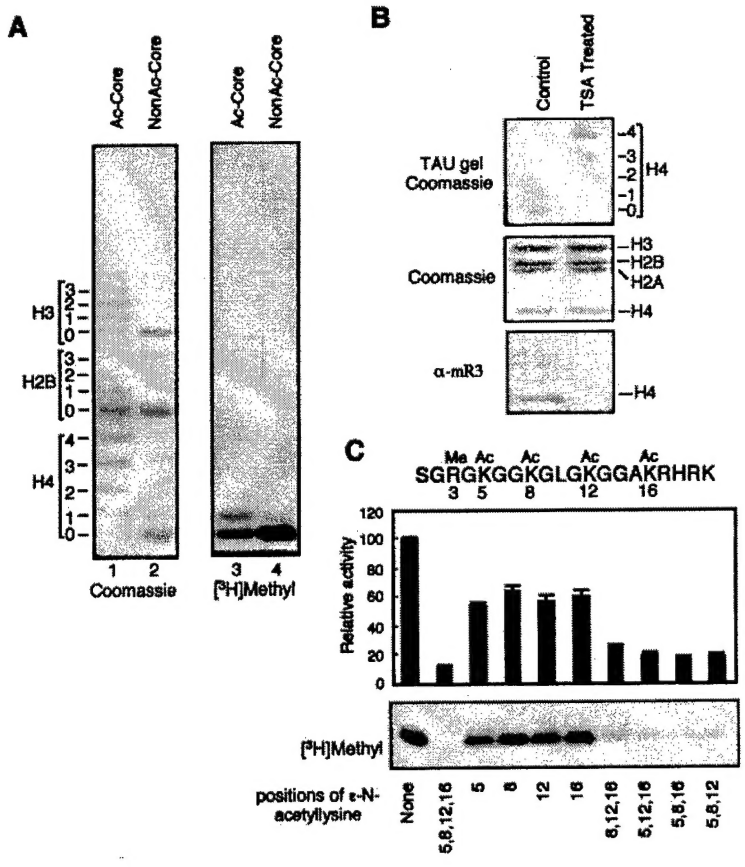

Fig. 4. Acetylation of H4 inhibits Arg 3 methylation by PRMT1. (**A**) Lysine acetylation inhibits H4-Arg 3 methylation in vitro. Hyper- (Ac) and hypoacetylated (Non-Ac) core histones purified from HeLa cells (*17*) were used as substrates for PRMT1 methylation (*10*). Different acetylated isoforms were resolved by a TAU gel and visualized by Coomassie staining. Methylation of different acetylated isoforms by PRMT1 was revealed by fluorogram. (**B**) Lysine acetylation inhibits H4-Arg 3 methylation in vivo. Core histones purified from untreated and TSA-treated (100 ng/ml final concentration) HeLa cells were analyzed by TAU gel (top panel), SDS-PAGE (middle panel), and Western blot (bottom panel). TAU gel reveals the acetylation state of H4, SDS-PAGE reveals equal loading, and Western blot reveals Arg 3 methylation state. (**C**) Comparison of the efficiency of PRMT1 to methylate different acetylated H4 peptides. Lysine residues that can be acetylated in vivo are indicated (top). Synthetic peptides that were not acetylated, monoed, triacetylated, and fully acetylated, respectively, were methylated with PRMT1 (*10*) and resolved by 20% SDS-PAGE before exposure to x-ray film (bottom). For quantification, the gel was cut and counted with scintillation counting. Results shown (middle) represent the average of two independent experiments with deviation.

not rule out the possibility that PRMT1 is an upstream regulator of an H4 Arg 3–specific HMT involved in H4 methylation through a methylation pathway similar to phosphorylation.

Having established that PRMT1 plays a critical role in Arg 3 methylation in vivo, we next sought to determine the function of this modification. Recent demonstration that methylation on Lys 9 of H3 inhibits Ser 10 phosphorylation (*4*) prompted us to ask whether Arg 3 methylation interferes with acetylation of lysine residues on H4 tails. To this end, we compared recombinant H4 that was either mock-methylated or PRMT1 methylated to serve as substrates for acetylation by p300 in the presence of [^{3}H]acetyl-CoA (*16*). Methylation of H4 by PRMT1 stimulated its subsequent acetylation by p300 (Fig. 3A). To confirm this result, samples equivalent to those analyzed in Fig. 3A were analyzed with a Triton-Acetic Acid-Urea (TAU) gel, which separates different acetylated histone isoforms. The results demonstrate that PRMT1-methylated H4 is a better substrate for p300 when compared with unmethylated H4, because all H4 molecules were acetylated (no 0 acetylated form) by p300 (Fig. 3B). However, under the same conditions, a fraction of the mock-methylated substrates still remains unacetylated (0 acetylated form). To determine which of the four acetylable lysine residues are affected by Arg 3 methylation, the acetylation status of samples analyzed above was examined by using acetylation site-specific antibodies. The results indicated that Arg 3 methylation facilitates K8 and K12 acetylation but has little affect on K5 or K16 acetylation (Fig. 3C).

To determine the effect of lysine acetylation on Arg 3 methylation, we purified both hyperacetylated and hypoacetylated core histones from HeLa cells (*17*) and used them as substrates for PRMT1 in the presence of [^{3}H]SAM. After methylation, samples were resolved in a TAU gel followed by Coomassie staining and autoradiography. Only un- and monoacetylated H4 isoforms were methylated to a detectable level, although nearly equal amounts of the different H4 isoforms were present in the methylation reaction (Fig. 4A, compare lanes 1 and 3). Because unacetylated H4 is the best substrate for PRMT1, when compared with different acetylated H4 isoforms (Fig. 4A), we concluded that acetylation on lysine residues inhibits H4 methylation by PRMT1. To determine whether this inhibition occurs in vivo, HeLa cells were treated with a histone deacetylase inhibitor, Tricostatin A (TSA), to induce hyperacetylation. Twelve hours after TSA treatment, core histones were isolated, and the methylation state of H4-Arg 3 was analyzed. Hypoacetylated H4 (untreated) had a higher amount of Arg 3 methylation when compared with hyperacetylated H4 (TSA treated), which had almost undetectable Arg 3 methylation (Fig. 4B). Therefore, hyperacetylation on lysine residues correlates with hypomethylation of H4 Arg 3. This result is consistent with the idea that acetylation on lysine residues inhibits its subsequent Arg 3 methylation, and it is also consistent with earlier studies demonstrating that H4 methylation preferentially occurs on unacetylated histones, whereas H3 methylation occurs preferentially on acetylated histones (*18*). Because H4 contains four lysine residues that can be acetylated (Fig. 4C, top panel), we investigated whether acetylation on any of the four sites would have a similar effect on Arg 3 methylation. To this end, synthetic H4 tail peptides that were not acetylated or were monoacetylated, triacetylated and fully acetylated, were used as substrates for PRMT1. Acetylation on any of the four lysines inhibited Arg 3 methylation by PRMT1 (Fig. 4C). However, acetylation on Lys 5 had the most effect. In addition, acetylation on different lysines seemed to have an additive inhibition effect. Consistent with results shown in Fig. 4A, triacetylated and fully acetylated peptides were severely impaired in serving as substrates for PRMT1 (Fig. 4C).

That Arg3 methylation enhanced lysine acetylation (Fig. 3) predicts that PRMT1 is likely to be involved in transcriptional activation. Indeed, PRMT1 has been shown recently to function as a coactivator of nuclear hormone receptors (*19*). However, its coactivator activity has not been linked to its HMT activity. To directly address the function of Arg 3 methylation on transcription, we introduced a single amino acid mutation (G80R) in the conserved SAM binding domain of PRMT1, which has been previously shown to impair its enzymatic activity (*20*). The ability of the mutant and wild-type PRMT1 to facilitate activation by androgen receptor (AR), which is known to use CBP/p300 as coactivators, was compared in chromatin context by using *Xenopus* oocytes as a model system (*21*). A mouse mammary tumor virus (MMTV) long terminal repeat (LTR)-based reporter was injected into the nuclei of *Xenopus* oocytes, and successful assembly of the reporter into chromatin was confirmed by micrococcal nuclease digestion (Fig. 5A). Ectopic expression of AR in *Xenopus* oocytes led to an agonist-stimulated activation of the reporter (Fig. 5B, compare lanes 2 and 3). Co-expression of PRMT1 further augmented the activation by AR (Fig. 5B, compare lanes 3 and 5). Significantly, the PRMT1(G80R) mutant has little coactivator activity when compared with wild-type PRMT1 (Fig. 5B, compare lanes 4 and 5 with lanes 6 and 7). Western blot analysis revealed that the differences in transcription were not due to differential expression of PRMT1 and PRMT1(G80R) or their effect on AR expression (Fig. 5B). We thus conclude that the HMT activity of PRMT1 is critical for its coactivator activity.

Our studies demonstrating the interplay between Arg3 methylation and lysine acetylation support the "histone code" hypothesis (*1*). We provided evidence that H4 Arg 3 methylation plays an important role in transcriptional activation. An H3-specific arginine methyltransferase CARM1 was also shown to function as a nuclear hormone receptor coactivator (*9*, *22*). In contrast, the heterochromatin-associated protein SUV39H1 was found to be an H3-specific methyltransferase (*4*), and methylation of Lys 9 by SUV39H1 serves as a binding site for the recruitment of the heterochromatin protein 1 (HP1) (*5*–*7*), suggesting that methylation of Lys 9 on H3 is likely involved in heterochro-

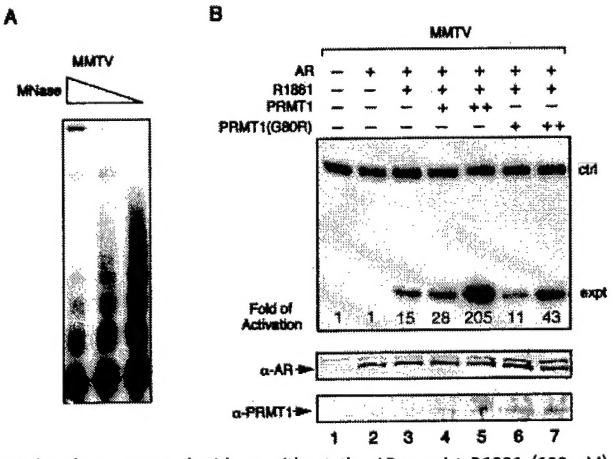

Fig. 5. The PRMT1 HMT activity is required for PRMT1 to function as a coactivator for AR. (**A**). The MMTV-LTR–based reporter injected into the nuclei of *Xenopus* oocytes was assembled into regularly spaced nucleosomes as revealed by Southern blot of a micrococcal nuclease digestion (MNase) assay (*11*). (**B**) Groups of *Xenopus* oocytes were injected with the MMTV-LTR reporter and the in vitro synthesized mRNAs encoding AR (100 ng/μl), PRMT1, or PRMT1(G80R) (100 ng/μl or 300 ng/μl) as indicated and were treated with or without the AR agonist R1881 (100 nM) overnight. The level of transcription from the reporter (expt) was analyzed by primer extension analysis of the total RNAs prepared from each group of oocytes and quantified by phosphor screen autoradiography (*11*). Folds of activation are shown below the primer extension product. The primer extension product from the endogenous histone H4 mRNA served as an internal control (ctrl). The expression levels of AR, PRMT1, and PRMT1(G80R) in each group of oocytes were analyzed by Western blot using an AR- or PRMT1-specific antibody, respectively.

matic gene silencing. Whether Arg 3 methylation helps the recruitment of specific histone acetyltransferases, such as p300, remains to be determined. As new HMTs responsible for the methylation of different histone arginine or lysine residues are identified, the functions of histone methylation on transcription and other processes involving chromatin will be revealed.

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23. One 100-mm plate of 293T cells (about 1.5×10^6) were transfected with 4 μg of empty pCDNA vector or pCDNA-PRMT1 by using the Effectene transfection reagent (Qiagen). Forty-eight hours after transfection, nuclei were isolated and core histones were purified by acid extraction and trichloroacetic acid precipitation.
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Identification of a Gene Associated with Bt Resistance in *Heliothis virescens*

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Transgenic crops producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used for pest control. Bt-resistant insect strains have been studied, but the molecular basis of resistance has remained elusive. Here, we show that disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion was linked to high levels of resistance to the Bt toxin Cry1Ac in the cotton pest *Heliothis virescens*. Monitoring the early phases of Bt resistance evolution in the field has been viewed as crucial but extremely difficult, especially when resistance is recessive. Our findings enable efficient DNA-based screening for resistant heterozygotes by directly detecting the recessive allele.

Field populations of the tobacco budworm *H. virescens*, a key pest of cotton and other crops in the Americas, have developed resistance to most classes of chemical insecticides. This species is the primary target of recently commercialized transgenic Bt cotton, which protects itself from insect damage by producing the insecticidal Cry1Ac toxin from *B. thuringiensis*. Concerns about Bt resistance led the U.S. Environmental Protection Agency to mandate a management plan, the "high-dose/refuge strategy" (*1*). It assumes that Bt cotton produces enough toxin to kill heterozygotes (with just one resistance allele) as well as

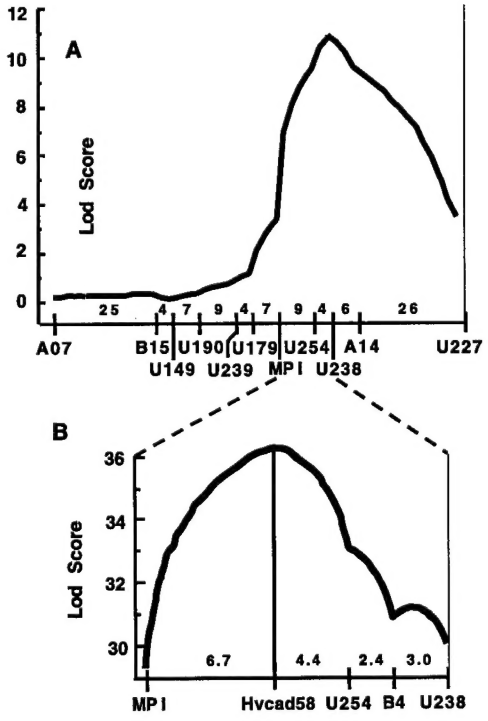

Fig. 1. QTL mapping of Cry1Ac resistance on linkage group 9 of *H. virescens*. (**A**) Resistance QTL lod (logarithm of the odds ratio for linkage) profile for initial scan of 105 cM on LG 9 spanned by 11 markers, based on 48 progeny of segregating backcross family D6. Marker order and spacing (in cM) was calculated by Mapmaker EXP 3.0 (*16*) and lod scores by Mapmaker QTL 1.9 (*17*). (**B**) Lod profile for fine-scale QTL mapping over the 16-cM region between MPI and U238, based on 268 progeny of nine segregating backcross families. The maximum lod score of 35.9 occurs at Hvcad58, which accounts for 46% of the trait variance. The resistance trait is the log of larval weight after 10 days of growth on 0.032 μg of Cry1Ac toxin per milliliter of diet (*3*).